



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C07K 14/31</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/48727</b> <b>(43) International Publication Date:</b> 24 December 1997 (24.12.97)
<b>(21) International Application Number:</b> PCT/SE97/01091 <b>(22) International Filing Date:</b> 18 June 1997 (18.06.97)  <b>(30) Priority Data:</b> 9602496-3      20 June 1996 (20.06.96)      SE  <b>(71)(72) Applicants and Inventors:</b> GUSS, Bengt [SE/SE]; Dag Hammarskjölds väg 238B, S-756 52 Uppsala (SE). NILSSON, Martin [SE/SE]; Dragarbrunnsgatan 60, S-752 30 Uppsala (SE). FRYKBERG, Lars [SE/SE]; Stabby Allé 7C, S-752 29 Uppsala (SE). FLOCK, Jan-Ingmar [SE/SE]; Sångarvägen 2, S-161 28 Bromma (SE). LINDBERG, Martin [SE/SE]; Sveriges Lantbruksuniversitet, Inst. för mikrobiologi, P.O. Box 7025, S-750 07 Uppsala (SE).  <b>(74) Agents:</b> ONN, Thorsten et al.; AB Stockholms Patentbyrå, Zacco & Bruhn (publ), P.O. Box 23101, S-104 35 Stockholm (SE).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>
<b>(54) Title:</b> NEW FIBRINOGEN BINDING PROTEIN ORIGINATING FROM COAGULASE-NEGATIVE STAPHYLOCOCCUS  <b>(57) Abstract</b> <p>A new fibrinogen binding protein or polypeptide originating from coagulase negative staphylococci, biotechnological methods for producing said protein or polypeptide having fibrinogen binding activity and a recombinant DNA molecule coding for said protein (or fragments thereof), and micro-organisms (including viruses) containing this recombinant DNA molecule. The present invention further comprises the therapeutic and diagnostic use of said protein and/or DNA, e.g. a diagnostic kit for determining the presence and/or type of coagulase negative staphylococci and a vaccine composition, comprising said protein or DNA.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## NEW FIBRINOGEN BINDING PROTEIN ORIGINATING FROM COAGULASE-NEGATIVE STAPHYLOCOCCUS

The invention relates to the field of gene technology and is concerned with recombinant DNA molecules, which contain a nucleotide sequence coding for a protein or polypeptide having fibrinogen-binding activity. Moreover the invention comprises micro-organisms (including viruses) containing the aforesaid molecules, and the use thereof in the production of the aforesaid protein or polypeptide and their use in biotechnology. Further, the present invention comprises diagnostic and therapeutic uses of said new protein, e.g. compositions for active and/or passive immunisation.

**Background of the invention**

During the last decade, the coagulase-negative staphylococci (CNS) have attracted an increasing attention. Along with the development of human and veterinary medicine, the number of susceptible hosts have increased. Advanced surgery, an increased use of bio-materials, medication with cytostatics, antibiotics and other drugs together with an increased frequency of antibiotic resistant strains of CNS have increased the susceptibility of the host. Concerning the veterinary importance of the CNS it is known that they can cause e.g. both sub-clinical and clinical inflammation in the bovine udder. The existence of bacteria that bind specifically to fibrinogen has been known for many years. The role of fibrinogen binding in the interaction process between the host and *Staphylococcus aureus* is still not clear but the fibrinogen-binding has been considered as one potential virulence factor of this species for instance in endocarditis (Moreillon et al 1995). No protein with fibrinogen binding properties has hitherto been described originating from CNS. However, the present invention describes the characterization and isolation of such protein using gene cloning. Furthermore, the invention describes different methods to measure the fibrinogen binding activity on cells of CNS and the use of this protein in biotechnology.

Generally, it might be difficult to obtain a homogeneous and a reproducible product if such a binding protein was prepared from staphylococcal cells directly. Moreover staphylococci are pathogenic and need complex culture media, which involves complications in large-scale cultures. There is thus a need for a new method for producing a fibrinogen binding protein (or fragments thereof).

**Summary of the invention**

The present invention discloses a new fibrinogen binding protein called FIG, a DNA molecule encoding said protein and applications for their use, according to the attached claims. Importantly, the present invention fills the long felt need of providing methods and means for diagnosing, type-determination, treatment and prevention of infections, caused by coagulase negative bacteria.

### Short description of the figures

The invention will be described in closer detail in the following, with support of the enclosed examples and figures, in which

Fig. 1 shows the adherence values as a function of fibrinogen coating concentration for the *S. epidermidis* strains 2, 19 and JW27 (Example 1A),

Fig. 2 shows percent inhibition for antibodies against fibrinogen, compared to antibodies against fibronectin (Example 1B),

Fig. 3 shows percent inhibition as a function of competing fibrinogen concentration (Example 1C),

Fig. 4 shows the protease sensitivity of adherence to fibrinogen (Example 1D),

Fig. 5 shows the inhibition of adherence by LiCl extract (Example 1E),

Fig. 6 shows the complete nucleotide sequence of the *fig* gene from *S. epidermidis* strain HB and the deduced amino acid sequence of the encoded protein. A putative ribosomal binding site (RBS) is underlined and a possible transcription termination hairpin loop is double underlined. A putative signal sequence (S) is indicated with an arrow and the translational stop codon with an asterisk. The start of the non-repetitive N-terminal region called A, harbouring the fibrinogen binding activity is indicated by an arrow. R indicates the highly repetitive region. The motif LPXTG involved in cell wall anchoring is indicated in bold characters and the membrane-spanning region is marked M (Example 3),

Fig. 7 shows a schematic drawing comparing the fibrinogen binding protein FIG of *S. epidermidis* and the clumping factor (ClfA) of *S. aureus*. The similarity, (%), of corresponding regions in the proteins is indicated in the figure between the two protein bars. S is the signal sequence; A, the non-repetitive region harbouring the fibrinogen binding activity; R, the diaminic acid residue repeat region; W the region proposed to be involved in cell wall anchoring and M, the transmembrane domain. The numbers indicated refer to the amino acid positions in respective proteins as shown in Figure. 6 and in reference (McDevitt et al., 1994) (Example 3),

Fig. 8 shows how GST-FIG fusion protein is captured to fibrinogen in a dose dependent way (Example 10),

Fig. 9 shows the decrease of bacterial binding as a function of GST-FIG fusion protein, GST or FIG (Example 11),

Fig. 10 shows the relative adherence as function of serum dilution for two pre immune sera and a serum against GST-FIG and FIG, respectively (Example 12), and

Fig. 11 shows the relative bacterial adherence as a function of serum dilution for, on one

hand, pre immune serum and, on the other hand, serum against GST-FIG (Example 12).

### Description of the invention

The present invention relates to a recombinant DNA molecule comprising a nucleotide sequence, which codes for a protein or polypeptide having fibrinogen-binding activity. The natural source of this nucleotide sequence is of course the *S. epidermidis* strain HB but with the knowledge of the nucleotide and deduced amino acid sequence presented here, the gene or parts of the gene can be isolated or made synthetically. In particular the knowledge of the deduced amino acid sequence for the part of the protein responsible for the fibrinogen binding activity can be used to produce synthetic polypeptides, which retain or inhibit the fibrinogen binding. These polypeptides can be labelled with various compounds such as enzymes, fluorescence, biotin (or derivatives of), radioactivity, etc and used e.g. in diagnostic tests such as ELISA- or RIA-techniques.

For production of a recombinant DNA molecule according to the invention a suitable cloning vehicle or vector, for example a phagemid, plasmid or phage DNA, may be cleaved with the aid of a restriction enzyme whereupon the DNA sequence coding for the desired protein or polypeptide is inserted into the cleavage site to form the recombinant DNA molecule. This general procedure is well known to a skilled person, and various techniques for cleaving and ligating DNA sequences have been described in the literature (see for instance US 4,237,224; Ausubel et al 1991; Sambrook et al 1989). Nevertheless, to the present inventors' knowledge, these techniques have not been used for the present purpose. If the *S. epidermidis* strain HB is used as the source of the desired nucleotide sequence it is possible to isolate said sequence and to introduce it into a suitable vector in manner such as described in the experimental part below or, since the nucleotide sequence is presented here, use a polymerase chain reaction (PCR)-technique to obtain the complete or fragments of the *fig* gene.

Hosts that may be used are, micro-organisms (which can be made to produce the protein or active fragments thereof), which may comprise bacterial hosts such as strains of e.g. *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus* sp., *Lactobacillus* sp. and furthermore yeasts and other eukaryotic cells in culture. To obtain maximum expression, regulatory elements such as promoters and ribosome binding sequences may be varied in a manner known per se. The protein or active peptide thereof can be produced intra- or extra-cellularly. To obtain good secretion in various bacterial systems different signal peptides could be used. To facilitate purification and/or detection the protein or fragment thereof could be fused to an affinity handle and /or enzyme. This can be done on both genetic and protein level. To modify the features of the protein or polypeptide thereof

the gene or parts of the gene can be modified using e.g. *in vitro* mutagenesis; or by fusion of other nucleotide sequences that encode polypeptides resulting in a fusion protein with new features.

The invention thus comprises recombinant DNA molecules containing a nucleotide sequence, which codes for a protein or polypeptide having fibrinogen-binding properties.

5 Furthermore the invention comprises vectors such as e.g. plasmids and phages containing such a nucleotide sequence, and organisms, especially bacteria as e.g. strains of *E. coli*, *B. subtilis* and *Staphylococcus* sp., into which such a vector has been introduced. Alternatively, such a nucleotide sequence may be integrated into the natural genome of the micro-organism.

10 The application furthermore relates to methods for production of a protein or polypeptide having the fibrinogen binding activity of protein FIG or active fragments thereof. According to this method, a micro-organism as set forth above is cultured in a suitable medium, whereupon the resultant product is isolated by some separating method, for example ion exchange chromatography or by means of affinity chromatography with the aid of fibrinogen bound to an insoluble carrier.

Vectors, especially plasmids, which contain the protein FIG encoding nucleotide sequence  
15 or parts thereof may advantageously be provided with a readily cleavable restriction site by means of which a nucleotide sequence, that codes for another product, can be fused to the protein FIG encoding nucleotide sequence, in order to express a so called fusion protein. The fusion protein may be isolated by a procedure utilising its capacity of binding to fibrinogen, whereupon the other component of the system may if desired be liberated from the fusion protein. This technique has  
20 been described at length in WO 84/03103 in respect of the protein A system and is applicable also in the present context in an analogous manner. The fusion strategy may also be used to modify, increase or change the fibrinogen binding activity of protein FIG (or part thereof) by fusion of other fibrinogen binding molecules.

The present invention also applies to the field of biotechnology that concerns the use of  
25 bacterial cell surface components as immunogens for vaccination against CNS infections. Immunisation using whole bacteria will always trigger a highly polyclonal immunresponse with a low level of antibodies against a given antigenic determinant. It is therefor preferable to use the protein, polypeptide or DNA according to the present invention for immunisation therapies. Notably, immunisation therapies can be conducted as so called passive and active  
30 immunisation. Passive immunisation using the inventive protein or DNA involves the raising of antibodies against the said protein or protein encoded by the administered DNA in a suitable host animal, preferably a mammal, e.g. a healthy blood donor or a cow, collecting and administering said antibodies to a patient. One preferred embodiment is passive immunisation.

of a patient prior to surgery, e.g. operations involving foreign implants in the body. Active immunisation using the inventive protein or DNA involves the administration of the said protein or DNA to a patient, preferably in combination with a pharmaceutically suitable immunostimulating agent. Examples of such agents include, but are not limited to the following: cholera toxin and/or derivatives thereof, heat labile toxins, such as *E. coli* toxin and similar agents. The composition according to the present invention can further include conventional and pharmaceutically acceptable adjuvants, well known to a person skilled in the art of immunisation therapy. Preferably, in an immunisation therapy using the inventive DNA or fractions thereof, said DNA is preferably administered intramuscularly, whereby said DNA is incorporated in suitable plasmide carriers. An additional gene or genes encoding a suitable immunostimulating agent can preferably be incorporated in the same plasmide.

Said immunisation therapies are not restricted to the above-described routes of administration, but can naturally be adapted to any one of the following routes of administration: oral, nasal, subcutaneous and intramuscular. Especially the oral and nasal methods of administration are potentially very promising, in particular for large-scale immunisations.

### Examples

#### Starting materials

#### Bacterial strains, phages and cloning vectors

*Staphylococcus epidermidis* strain HB was obtained from Dr Åsa Ljungh, Lund, Sweden.

*E. coli* strain TG1 and strain MC1061 were used as bacterial host for construction of the library and production of the phage stocks. The *E. coli* phage R408 (Promega, Madison, WI, USA) was used as helper phage.

The phagemid vector pG8H6 used is described Jacobsson and Frykberg (1996).

All strains and plasmid- or phagemid- constructs used in the examples are available at the Department of Microbiology at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

#### Buffers and media

*E. coli* was grown on LB (Luria Bertani broth) agar plates or in LB broth (Sambrook et al 1989) at 37°C. In appropriate cases the LB medium was supplemented with glucose to a final conc. of 2%. Ampicillin was in appropriate cases added to the *E. coli* growth media to a final conc. of 50 µg/ml. Staphylococci were grown at 37°C on blood agar-plates (containing 5% final conc. bovine blood) or in Tryptone Soya Broth (TSB obtained from Oxoid, Ltd Basingstoke, Hants., England) PBS: 0,05M sodium phosphate pH 7.1, 0.9 % NaCl. PBS-T: PBS supplemented with TWEEN 20

to a final conc. of 0.05 %.

#### Preparation of DNA from staphylococci and streptococci

Strains of *S. epidermidis* or *S. aureus* were grown overnight in TSB. Next morning the cells were harvested and the chromosomal DNA prepared according to Löfdahl et al (1983).

5 Chromosomal DNA from streptococci has earlier been described in WO 95/07300.

#### Proteins and other reagents

Human fibrinogen was obtained from (IMCO Ltd, Stockholm, Sweden). Human serum albumin (HSA), fibronectin, IgA, lactoferrin and transferrin were obtained from Sigma, St. Louis, USA). Bovine serum albumin (fraction V, ria grade) was obtained from USB (cat. no.10868).  
10  $\alpha_2$ macroglobulin ( $\alpha_2$ M) and collagen type I were obtained from Boehringer, Mannheim, Germany). Vitronectin was obtained from Bional, Tartu, Estonia and human IgG from Kabi, Stockholm, Sweden. Elastin was obtained from ICN Pharmaceuticals Inc. CA, USA and pepsin from KEBO LAB, Stockholm, Sweden.

DNA probes were labelled with  $\alpha^{32}$ P-ATP by a random-priming method (Multiprime DNA  
15 labelling system; Amersham Inc, Amersham, England)

Nitrocellulose (NC) filters (Schleicher & Schüll, Dassel, Germany) were used to bind DNA in hybridisation experiments or proteins in Western-blot techniques.

In order to analyse protein samples by native or sodium dodecyl sulphate -polyacrylamid gel electrophoresis (SDS-PAGE) the PHAST-system obtained from Pharmacia LKB  
20 Biotechnology, Uppsala, Sweden was used according to the supplier's recommendations.

Oligonucleotides used were synthesised by Pharmacia (Uppsala, Sweden).

Micro Well plates (MaxiSorp, Nunc, Copenhagen, Denmark) were used in the panning experiment. Plasmid DNA was prepared using Wizard Minipreps (Promega) and the sequence of the inserts was determined as described by Jacobsson and Frykberg (1995). The sequences  
25 obtained were analysed using the PC-gene program (Intelligenetics, Mountain View, CA, USA)

#### Routine methods

Methods used routinely in molecular biology are not described such as restriction of DNA with endonucleases, ligation of DNA fragments, plasmid purification etc since these methods can be found in commonly used manuals (Sambrook et al., 1989, Ausubel et al., 1991). Ligation  
30 reactions were performed using Ready-To-Go T4 DNA Ligase (Pharmacia, Uppsala, Sweden). For polymerase chain reaction amplification the Gene Amp™ kit, obtained from Perkin Elmer Cetus, was used. Sequence reactions were performed using "Sequenase, version 2.0" kit (United States Biochemical Corporation, Cleveland, Ohio, USA). Alternatively the ABI PRISM Dye Terminator



Cycle Sequencing Ready Reaction Kit was used and the samples analysed using the Applied Biosystems 373A DNA Sequencer.

Example 1: The adherence of *Staphylococcus epidermidis* to immobilised fibrinogen and investigation of the nature of the binding mechanism (A-E)

- 5        Strains of *Staphylococcus epidermidis* isolated from cases of peritonitis were grown on Blood agar plates at 37°C overnight. The bacteria from one plate was harvested with 5 ml phosphate buffered saline (PBS), washed once, and the optical density (OD) was adjusted to 1.0.

(A) Bacterial adherence

- 10        Fibrinogen was dissolved in PBS at 10 mg/ml and added in serial 3-fold dilution to microtiter wells (Nunc), from top to bottom. The plates were incubated overnight at room temperature (RT). To cover uncoated plastic sites the plates were coated with 2% bovine serum albumin for 1 hour at 37°C. The plates were washed with PBS with 0.05% Tween 20 (PBST). Next, bacteria were added in serial 2- fold dilution in PBST, from left to right, to the fibrinogen coated microtiter plates. Bacterial adherence was allowed for 2 hours at 37°C or at 4°C overnight.
- 15        Non-adherent bacteria were washed off and the bound bacteria were air-dried. The crosswise dilution of both fibrinogen and bacteria allows estimation of bacterial binding both as a function of fibrinogen concentration and of amount of bacteria. Determination of bacterial adherence was done by optical reading using a microtiter plate reader at A 405. The turbidity and light scatter caused by bound bacteria results in a reading ranging from 0.00 to 0.20. An example of adherence values as a
- 20        function of fibrinogen coating concentration is shown in Figure 1 for three different strains (2, 19 and JW27). These conditions for adherence determination were used in the following experiments.

(B) Adherence blocking by antibodies against fibrinogen

- 25        In a modification of the experiment performed above, antibodies against fibrinogen (anti Fg) (Sigma) were added 1 hour prior to addition of bacteria (OD=1.0) to the immobilised fibrinogen. As a control, antibodies against fibronectin (anti Fn) (Sigma) were added in a separate experiment. Figure 2 shows that antibodies against fibrinogen (circles) inhibited adherence better than antibodies against fibronectin could (squares). The mean values and standard errors from three separate experiments are shown.

(C) Adherence blocking by soluble fibrinogen

- 30        Soluble fibrinogen was added to the bacteria at concentrations indicated in Figure 3 and incubated for 1 hour at 37°C before addition to plates coated with fibrinogen as described above. Adherence of *S. epidermidis* strain 19 (filled circles) was inhibited to around 30%. As a control,

inhibition of *Staphylococcus aureus* strain Newman was measured in a similar experimental set-up (open circles). Mean values and standard errors from three separate experiments are shown. Although significant inhibition of adherence of *S. epidermidis* was obtained, inhibition of *S. aureus* was more pronounced.

5 (D) Reduction of binding after protease treatment of bacteria

Bacteria were treated for 30 minutes at 37°C with protease K, at concentrations indicated in Figure 4, prior to addition to immobilised fibrinogen. Protease treated bacteria were extensively washed after protease treatment to avoid protease digestion of the immobilised fibrinogen. Four different strains of *S. epidermidis* (2, 19, 269 and HB) and *S. aureus* (strain Newman) were used in this experiment. All strains tested showed sensitivity to protease treatment; thus the adherence to fibrinogen depends on a surface protein.

10 (E) Adherence blocking by LiCl extract of *S. epidermidis*

*S. epidermidis* cells, grown and harvested as described above, were treated with 1M LiCl at 40°C for 2 hours with continuous gentle stirring. The bacteria were centrifuged and the bacteria-free supernatant was filtered and dialysed against PBS. Surface associated proteins bound to the cells by hydrophobic interactions are thereby released. This LiCl extract, presumably containing a fibrinogen binding protein, was used to inhibit adherence of *S. epidermidis* to immobilised fibrinogen in the following way: LiCl extract at various dilutions was added to the immobilised fibrinogen and incubated for 1 hour at 37°C. The plates were washed and bacteria added for adhesion testing. Fig. 5 shows that adherence was better the more the LiCl extract was diluted; i.e. an adhesion-inhibitory compound is present in the LiCl extract. Two independent experiments are shown.

20 Example 2: Isolation of a clone expressing fibrinogen binding activity

A gene library of *S. epidermidis* strain HB was produced in a manner as described by Jacobsson and Frykberg (1996). Staphylococcal DNA was randomly fragmented by sonication. The library resulted in  $4 \times 10^7$  independent clones, which after amplification had a titer of  $2 \times 10^{10}$  cfu/ml. Two hundred microlitres of the library were added to each of three fibrinogen coated wells and incubated for 4 hour at room temperature (RT). The wells were washed extensively with PBS-T and once with 50mM Na-citrate/140 mM NaCl, pH 5.4. Finally, the bound phages were eluted stepwise in the same buffer with decreasing pH (3.4 and 1.8). The eluates from the three wells were neutralised with 2 M Tris-HCl, pH 8.6. Aliquots of the eluates were used to infect *E. coli* TG1 cells, which thereafter were grown overnight on LA plates containing glucose and ampicillin. The

colonies (obtained after infection of TG1 cells with the phage and eluted at pH 3.4 and 1.8 in the primary panning) were collected by resuspension in LB medium and infected with helper phage R408 [ $10^{10}$  plaque-forming units (pfu)] for production of enriched phage stocks. Thereafter, the infected bacteria were mixed with 4 ml 0.5% soft agar and poured on one LA plate with ampicillin.

- 5 After incubation over night  $37^{\circ}\text{C}$  the phages were collected as described by Jacobsson and Frykberg (1996). The resulting phage stock was repanned against fibrinogen as described above. The result presented in Table 1. shows that there is an enrichment of clones having affinity to fibrinogen.

10 Table 1.

	<i>Panning</i>	<i>Ligand</i>	
		Fibrinogen	IgG
	1st Wash	$1.6 \times 10^3$ cfu/ml	-
15	pH 5.4	$1.6 \times 10^3$ cfu/ml	-
	pH 3.4	$2.1 \times 10^3$ cfu/ml	-
	pH 1.8	$7.0 \times 10^3$ cfu/ml	-
	2nd Wash	$1.2 \times 10^3$ cfu/ml	$2.2 \times 10^2$ cfu/ml
20	pH 5.4	$4.4 \times 10^3$ cfu/ml	$6.2 \times 10^2$ cfu/ml
	pH 3.4	$4.3 \times 10^4$ cfu/ml	$1.4 \times 10^3$ cfu/ml
	pH 1.8	$2.0 \times 10^3$ cfu/ml	$8.0 \times 10^2$ cfu/ml

### Example 3: DNA sequencing and sequence analysis

- 25 Eight colonies coming from the second panning (pH 3.4) against fibrinogen described in Example 2 were chosen for further studies. Phagemid DNA from these colonies was prepared and partially sequenced. Seven of the clones seemed to contain the same insert. One of these seven clones called pSE100 was chosen for further studies. Purified phagemid DNA from the clone pSE100 was analysed by restriction mapping which revealed that the phagemid contained an insert
- 30 of  $\sim 1.8$  kilo base pair (kb). The nucleotide (nt) sequences of the complete inserts of pSE100 were determined and the nt and deduced amino acid (aa) sequences were analysed using the PC-gene program. This analysis revealed that the insert of pSE100 contains an open reading frame of 1.745 nt (sequence list). Thus the insert encodes a 581 aa protein, termed protein FIG (and the

corresponding gene termed *fig*), with a calculated molecular mass of ~65 kDa (sequence list). Furthermore, the sequence analysis show that the insert of pSE100 is in the correct reading frame with the vector sequences in the 5'- and 3'-ends. This means that the insert gives rise to a fusion with the *pel* leader and the *myc* tail (sequence list) and that the native 5'- and 3'-ends of the *fig* gene is not present in the pSE100 clone.

To obtain the missing 5' and 3' end of the *fig* gene a Southern blot analysis was performed using chromosomal DNA from strain HB digested with various restriction enzymes. The probe was prepared as follows; two oligonucleotides (5'-CAACAACCATCTCACACAAC3' and 5'-CATCAAATTGATATTTCCCATC3') were used to PCR amplify a ~1.3kb fragment from the insert of pSE100. The PCR generated fragments were 32P-labelled using random priming. After hybridisation using stringent conditions the NC-filter was washed and subjected to autoradiography. The result showed that the XbaI cleavage gave a single band in size of ~6 kb. The corresponding fragment was subsequently ligated into XbaI digested pUC18 vector. After transformation clones harbouring the ~6 kb XbaI-fragment were identified by colony hybridisation using the same probe as in the Southern blot experiment. One such clone, called pSE101 was chosen for further studies. DNA sequence analysis showed that the *fig* gene consist of an open reading frame of a 3291 nt, encoding a protein, called FIG of 1097 aa with a calculated molecular mass of ~119 kDa (Figure 6). The FIG protein consist of several typical features found among Gram-positive cell surface bound proteins, like a N-terminal signal sequence and a C-terminal aa motif LPDTG, followed by a stretch of 17 hydrophobic aa ending in a stretch of charged aa (Figure 6). Following the signal sequence, there is a region, called A of 773 aa. The insert of pSE100 contains the sequence corresponding to residue 75 to 656 of the A region (Figure. 7). The A region is followed by a highly repetitive region of 216 aa composed of tandemly repeated aspartic acid and serine residues, called R (Figure 6 and 7). The dipeptid region consist of an 18 bp sequence unit (consensus of GAX TCX GAX TCX GAX AGX) repeated 36 times. The 18 bp sequence is almost maintained perfect throughout the whole R region except for the second unit which is truncated, consisting of only 12 of the 18 bp and the 3' end of the region where the consensus sequence is slightly disrupted (units 32, 34 and 36). The changes in the later units also result in an amino acid exchange which disrupt the DS repeat.

Using the deduced amino acid sequence of protein FIG protein databases were screened for sequence similarities. Interestingly, the search showed that the highest score obtained was for the clumping factor (ClfA) of *S. aureus* (Figure 7). This protein binds fibrinogen and has been shown to promote aggregation of bacteria in the present of plasma. Beside similarities in the N- and

C-terminal part encoding the signal sequence and the cell membrane spanning domain, respectively the most obvious similarity with the clumping factor is the repetitive R region. In both ClfA and FIG protein, the DS repeat region is encoded by the same 18 bp consensus unit. Comparing the nucleotide sequences of *fig* and *clfA* shows that the R regions have an extensive homology. In addition, protein FIG also shows homology to ClfA in the A region, the non-repetitive fibrinogen binding domain (Figure 7).

Example 4: Properties of the fibrinogen binding protein encoded from pSE100

A) Specificity of the fibrinogen binding

The phagemid pSE100 was electroporated into competent *E. coli* TG1 cells. After growth over night on a LA plate (containing ampicillin and glucose) one colony containing pSE100 was grown over night and infected with the helper phage R408 for production of an enriched phage stock. The resulting phage stock containing recombinant phages expressing the insert of pSE100 had a titer of  $3 \times 10^9$  cfu/ml. The phage stock of pSE100 was used to pan against 13 different proteins coated in microtiter wells and to one uncoated well. To each well containing the respective protein (or to the uncoated well) 200  $\mu$ l of the phage stock of pSE100 was added. After panning for three hours at RT under gentle agitation the wells were washed extensively, using PBST and a sample of the last wash was collected. The bound phages were eluted with Na-Citrate buffer pH 1.8. The eluted samples were immediately neutralised using 1M Tris-HCl pH 8.6. The eluted phages and the phages from the wash were allowed to separately infect *E. coli* TG1 cells and after infection, the cells were plated on LA plates containing ampicillin and glucose. The plates were incubated over night at 37°C and the frequency of colonies was counted. The result of this experiment is presented in Table 2 which shows the fibrinogen binding specificity of the protein expressed by pSE100.

Table 2.

Ligand	Wash	Eluate pH 1.8
Fibrinogen	$1.1 \times 10^4$ cfu/ml	$1.4 \times 10^7$ cfu/ml
$\alpha_2$ M	$2.0 \times 10^2$ cfu/ml	$2.0 \times 10^3$ cfu/ml
5 BSA	$<10^2$ cfu/ml	$8.0 \times 10^2$ cfu/ml
Collagen type I	$6.0 \times 10^2$ cfu/ml	$1.2 \times 10^3$ cfu/ml
Elastin	$8.0 \times 10^2$ cfu/ml	$5.2 \times 10^3$ cfu/ml
Fibronectin	$6.0 \times 10^2$ cfu/ml	$2.4 \times 10^4$ cfu/ml
HSA	$8.0 \times 10^2$ cfu/ml	$2.2 \times 10^3$ cfu/ml
10 IgA	$6.0 \times 10^2$ cfu/ml	$6.8 \times 10^4$ cfu/ml
IgG	$4.0 \times 10^2$ cfu/ml	$4.4 \times 10^3$ cfu/ml
Lactoferrin	$6.0 \times 10^2$ cfu/ml	$8.2 \times 10^3$ cfu/ml
Pepsin	$1.8 \times 10^2$ cfu/ml	$3.7 \times 10^4$ cfu/ml
Transferrin	$2.0 \times 10^2$ cfu/ml	$2.4 \times 10^3$ cfu/ml
15 Vitronectin	$<10^2$ cfu/ml	$2.2 \times 10^3$ cfu/ml
Plastic	$2.4 \times 10^3$ cfu/ml	$9.0 \times 10^3$ cfu/ml

(B) Inhibition experiment

The pSE100 phage stock was diluted to a titer of  $\sim 5 \times 10^6$  cfu/ml. Of this phage solution  
 20 samples (180  $\mu$ l) were taken and separately incubated for one hour with different concentrations of  
 fibrinogen, BSA or IgG before transferred to fibrinogen coated microtiter wells. After panning for  
 three hours at RT under gentle agitation, the wells were washed extensively using PBST. The  
 bound phages were eluted with Na-Citrate buffer pH 1.8. The eluted samples were immediately  
 neutralised using 1M Tris-HCl pH 8.6. The eluted phages were allowed to infect *E. coli* TG1 cells  
 25 and after infection, the cells were plated on LA plates containing ampicillin and glucose. The plates  
 were incubated over night at 37°C and the frequency of colonies was counted. The result of this  
 experiment is presented in Table 3, which shows that the binding to fibrinogen is inhibited by  
 fibrinogen but not with the other tested proteins.

Table 3

Conc. of different		Soluble ligands		
ligands ( $\mu\text{g/ml}$ )		<i>Fibrinogen</i>	<i>BSA</i>	<i>IgG</i>
5	0	$7.6 \times 10^4$ cfu/ml	$7.6 \times 10^4$ cfu/ml	$7.6 \times 10^4$ cfu/ml
	0.1	$4.4 \times 10^4$ cfu/ml	$7.0 \times 10^4$ cfu/ml	$6.2 \times 10^4$ cfu/ml
	1	$3.6 \times 10^4$ cfu/ml	$9.3 \times 10^4$ cfu/ml	$9.0 \times 10^4$ cfu/ml
	10	$1.5 \times 10^4$ cfu/ml	$6.3 \times 10^4$ cfu/ml	$7.8 \times 10^4$ cfu/ml
	100	$3.8 \times 10^3$ cfu/ml	$6.4 \times 10^4$ cfu/ml	$7.3 \times 10^4$ cfu/ml
10	1000	$3.0 \times 10^2$ cfu/ml	$6.9 \times 10^4$ cfu/ml	$7.6 \times 10^4$ cfu/ml

Example 5: Western blot experiment

*E. coli* cells of strain TG1 and MC1061 containing pSE100 were grown in LB (containing ampicillin and glucose) over night at 37°C. The next morning the cells were harvested by centrifugation, resuspended in LB (containing ampicillin, glucose and 0.1 M IPTG and further incubated at 37°C. Twelve hours later the cells were harvested by centrifugation and both the cells and the supernatant were taken care of. Four volumes of acetone were added to the supernatant and the resulting precipitate was collected by centrifugation, air-dried and resuspended in ice-cold PBS. Prior to electrophoresis the cells and the precipitate from the supernatant were resuspended separately in a sample buffer containing 2.5% SDS and 5% beta-mercaptoethanol and boiled for two minutes. After denaturation the samples were analysed run under reducing conditions using the PHAST-system (Pharmacia) on a 8-25% gradient gel using SDS-buffer strips. After the electrophoresis was completed a NC-filter previously soaked in PBS was put on the gel and the temperature raised to 45°C. After ~45 minutes the NC-filter was wetted with 1 ml PBS, gently removed and placed in 15ml PBS containing 0.1% Tween 20 solution (PBST 0.1%) for 30 minutes in RT (under gentle agitation and with two changes of PBST 0.1% solution). After the last change of PBST 0.1% fibrinogen was added to a final conc. of 20ng/ml and the filter was incubated for four hours at RT under gentle agitation. The filter was subsequently washed for 3x10 minutes using PBST0.1% and HRP-conjugated rabbit anti-human fibrinogen antibodies (DAKO code A 080, diluted 1:500 in PBST 0.1%) were added and the filter was incubated for 1 hour at RT under gentle agitation. After washing the filter 3x10 minutes using PBST 0.1% the bound fibrinogen was visualised by transferring the filter to a solution containing a substrate for the horse radish

peroxidase (6 ml 4-chloro-1-naphtol (3 mg/ml in methanol) + 25 ml PBS + 20  $\mu$ l  $H_2O_2$ ). The result showed that a fibrinogen binding protein was found in both types of samples (cells and growth media) in both *E. coli* cells harbouring pSE100, while no such protein was found in the control cultures of *E. coli* TG1 and MC1061. The fibrinogen binding protein expressed from the pSE100 was in the approximate size as expected from the deduced amino acid.

Example 6: The occurrence of the *fig* gene and the use of *fig* gene to identify *S. epidermidis* in diagnostic test

Purified chromosomal DNA from *S. aureus* strain 8325-4, *Streptococcus equi* subsp. *equi* strain 196 and subspecies *zooepidemicus* strain Z5, *Streptococcus pyogenes* strain 2-1047, *Streptococcus dysgalactiae* strain 8215 were cleaved using the restriction enzyme *EcoRI*. The cleaved samples were run on an 0.8% agarose-gel together with chromosomal DNA from *S. epidermidis* strain HB cleaved with various restriction enzymes. After the electrophoresis was completed, the separated DNA fragments were transferred to a NC-filter using the Vacuum blotting system from Pharmacia. After the transfer the filter was hybridised under stringent conditions (in a solution containing 6xSSC, 5xDenhart, 0.5% SDS at 65°C) using a probe designed based on the nucleotide sequence of the insert of pSE100. This probe had earlier been prepared as follows, two oligonucleotides: (5'-AGGTCAAGGACAAGGTGAC-3' and 5'-CAACAACCATCTCAC ACAAC-3') were ordered (Pharmacia) and used as a primer pair in a PCR (25 cycles of 94°C 1 minute, 50°C 30 seconds, 72°C 1 minute using an Perkin Elmer Cetus Thermal Cycler 480) to amplify an ~150 bp fragment of the insert of pSE100. The amplified material was run on an agarose gel and the ~150 bp fragment was purified and radioactively labelled using  $^{32}P$ -dATP and the Multiprime DNA labelling system (Amersham). The filter was hybridised over night and subsequently washed in a washing solution (0.2% SSC, 0.1% SDS) at 60°C and autoradiographed. The result showed that no hybridisation was detected in the samples originating from streptococci and *S. aureus* while hybridisation occurred to the samples coming from the *S. epidermidis* strain HB.

To investigate the occurrence of the *fig* gene in other strains of *S. epidermidis* the following PCR reaction was set up. Chromosomal DNA from 13 different clinical isolates of *S. epidermidis* was used as templates. The same primers and the same PCR conditions as described above were used. The result showed that an amplified product of ~150 bp could be detected (using a 2% agarose gel) in all strains of *S. epidermidis* but not in the control samples original containing chromosomal DNA from *S. aureus* and *S. pyogenes*.



Example 7: A PCR amplification assay for analysis of corresponding DS repeat regions from various isolates of *S. epidermidis*

McDevitt and Foster (Microbiology, 1995,141:937-943) have shown that the DS repeat region in various isolates of *S. aureus* strains may differ considerable. To investigate if the DS repeat region in *S. epidermidis* also varies in size between different isolates following experiment was performed. A pair of primers (5'CCGATGAAAATGGAAAGTATC3' and 5'TCCGTTATCTATACTAAAGTC3') hybridising on the 5' and 3' side, respectively, of the DS repeat region of protein FIG were used to PCR amplify the corresponding region in 11 different isolates of *S. epidermidis*. The amplification was performed as follows, after initial denaturation for 1 min. at 95°C a cycle started with a denaturation step for 30 sec. at 95°C, followed by an annealing time of 1 min. at 50°C and a elongation period of 2 min. at 72°C. The cycle was repeated 25 times and ended in an final elongation period of 7 min. at 72°C. The PCR products representing the DS region of respective strain were analysed by agarose-gel electrophoresis. The result showed that one band of various length was present in each sample. The conclusion from this is that this type of method can be used as a diagnostic test to get a "fingerprint" of a particular strain. This might be useful in e.g. tracing a the origin of an infection.

Example 8: The use of the DS fragment of strain HB to identify other homologous genes in coagulase-positive and -negative staphylococci

A DNA fragment consisting of the DS repeat region was constructed as follows. One pair of oligonucleotide primers (5'ACTGATCATGATGACTTTAGT 3' and 5'TCCGTTATCTAT ACTAAAGTC3') was used to PCR amplify the DS region of strain HB using the same conditions as described above. The amplification resulted in a ~700 bp fragment which was radioactively (<sup>32</sup>P) labelled using random priming. This probe was used in a Southern blot analysis using chromosomal DNA (cleaved with EcoRI) from various species of *staphylococci* (*S. aureus*, *S. epidermidis* strain HB, *S. haemolyticus* strain 789 and strain SM131, *S. lugdunensis*, *S. schleiferi*, *S. intermedius*, *S. lentus*, *S. sciuri*, *S. carnosus*, *S. saprophyticus* and *S. hyicus*).

The hybridisation was performed under stringent conditions at 65°C over night. The next day the filter was washed at 65°C, using 2XSSC following autoradiography. The result showed that at least one specific band was present for the following species; *S. aureus*, *S. epidermidis* strain HB, *S. haemolyticus* strain 789 and strain SM131, *S. lugdunensis*, *S. intermedius*, *S. sciuri*, *S. carnosus* (weak signal) and *S. hyicus*. This result shows, that it is possible to clone and identify the corresponding regions in these species.

### Example 9: Production of GST-FIG

By polymerase chain reaction, a DNA fragment was amplified encoding a portion of the fibrinogen binding protein. Upper primer was GCGGATCCAATCAGTCAATAAACA CCGACGAT and lower primer was CGGAATTCTGTTCGGACTGATTTGGAAGTTCC.

5 Amplification was done for 30 cycles at 94°C 30 seconds, 60°C 30 seconds, 72°C 2 minutes beginning with 94°C for 4 minutes and ending with 72°C for 4 minutes. The amplified fragment was digested with EcoRI and Bam HI. Plasmid pGXT-4T (Pharmacia, Uppsala, Sweden) was digested with EcoRI and Bam HI, mixed with the digested fragment and the mixture ligated using T4 DNA ligase according to standard procedures. The ligated DNA was transformed into *E. coli* strain TG1.

10 A transformant was isolated with a plasmid encoding a fusion protein composed of glutathione thio transferase and fibrinogen binding protein. The protein was purified with the vector plasmid according to Pharmacia's instructions. The purified GST-FIG protein was subjected to Western affinity blot. It was run on polyacrylamide gel electrophoresis, transferred to nitrocellulose paper by passive diffusion, the paper treated with fibrinogen (5 µg/ml) for 2 hours at room temperature,

15 followed by rabbit anti fibrinogen antibodies conjugated to HRP. A band corresponding to a molecular weight of approx. 100 kDa was seen. Omitting fibrinogen in a control experiment displayed no band.

### Example 10: Demonstration of binding of GST-FIG to stationary phase fibrinogen

Microtiter wells were coated with human fibrinogen (Sigma Chemicals Co.) at a

20 concentration ranging from 2.5 to 20 µg/ml at room temperature overnight. The plates were aftercoated with 2% bovine serum albumin (BSA) for one hour at 37°C. The microtiter plates were washed three times and GST-FIG was added to the wells at concentrations of 25, 50 or 100 µg/ml (indicated by the three separate lines in Fig. 8) and the plates incubated for two hours at 37°C. Capture of GST-FIG to the fibrinogen layer was, after washing, detected by antibodies (diluted

25 1000 times) raised in a rat against His-FIG. Binding of antibodies was, after washing, detected with rabbit anti rat IgG antibodies conjugated with HRP. The substrate for HRP was OPD tablets (Dakopatts) with H<sub>2</sub>O<sub>2</sub>. Colour reaction was measured at 495 nm. Fig. 8 shows that GST-FIG is captured to fibrinogen in a dose dependent way.

### Example 11: Inhibition of *S. epidermis* adherence to fibrinogen by FIG

30 Fibrinogen at 2 µg/ml was used to coat microtiter wells overnight at room temperature and aftercoated as above. GST-FIG fusion protein, GST or FIG was added at concentrations indicated in Fig. 9. Radioactively labelled bacteria was added immediately after, and incubated at 37°C for

two hours. Decrease of bacterial binding as a function of GST-FIG fusion protein, GST or FIG is shown in Fig. 9. The symbols in Fig. 9 are the following: squares – inhibition by GST-FIG (mean and SE of five independent experiments are shown); triangles – inhibition by GST carrier protein; circles – inhibition by FIG after thrombin digestion. Only the fusion protein and FIG molecules could inhibit binding.

Radioactive labelling of bacteria was obtained by growing them in the presence of tritiated thymidine (20 $\mu$ Ci/ml, specific activity 81 Ci/mmol) for 5 hours in LB.

Cleavage of GST-FIG was achieved by adding thrombin and incubating at 37°C for 2 hours.

#### Example 12: Inhibition of *S. epidermidis* adherence to fibrinogen by antibodies against GST-FIG and FIG

Fibrinogen at 2 mg/ml was used to coat microtiterwells overnight at room temperature and aftercoated as above. Radiolabelled *S. epidermidis* were incubated with different dilutions of sera for 1 hour at 37°C. The bacteria - serum mixtures were then added to the wells and adherence was allowed to take place for two hours at 37°C. Non adherent bacteria were washed away and the amount of adherent bacteria were determined as in example 11 above. Four serum samples were used: 1) Serum from before immunisation from rat No 1. 2) Serum from before immunisation from rat No 2. 3) Serum from rat No 1 immunised with GST-FIG. 4) Serum from rat No 2 immunised with FIG generated by thrombin cleavage. From Figure 10 it can be seen that adherence is reduced after incubation with sera against FIG or against the GST-FIG fusion protein. With relative adherence of 1.0 is meant the adherence obtained after incubation of the radiolabelled bacteria with phosphate buffered saline.

The experiment was repeated, and data from adherence blocking, using sera taken before immunisation and serum taken after immunisation with GST-FIG is shown in Figure 11.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

#### **References**

Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) (1991) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Intersciences,

New York.

Bodén, M.K. and Flock, J.-I. (1995) J.Clin.Microbiol.33: 2347-2352. Incidence of the highly conserved fib gene and expression of the fibrinogen binding (Fib) protein among clinical isolates of *Staphylococcus aureus*.

- 5 Jacobsson, K. and Frykberg, L. (1995) Cloning of ligand-binding domains of bacterial receptors by phage display. BioTechniques 18:878-885

Jacobsson, K. and Frykberg, L. (1996) Phage display shotgun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. BioTechniques 20:1070-1081.

- 10 Löfdahl, S., Guss, B., Uhlén, M., Philipson, L. and Lindberg, M. (1983) Proc. Natl. Acad. Sci. USA 80:697-701.

McDevitt, D., Francois, P., Vaudaux, P. and Foster, T.J. (1994) Mol. Microbiol. 11:237-248.

Moreillon, P., Entenza, J.M. Francioli, P., McDevitt, D., Foster, T.J., Francois, P. and Vaudaux, P. (1995) Infect. Immun. 63:4738-4743.

- 15 Sambrook, J., Fritsh, E.F. and Maniatis, T. (1989) Molecular cloning, A laboratory manual, second ed., Cold Spring Harbour Laboratory Press, New York.

Wadström, T. and Rozgony, F. (1986) Virulence determinants of coagulase-negative staphylococci pp 123-130. In "Coagulase-negative staphylococci" Eds. Mårdh, P.-A. and Schleifer, K.H. Almquist & Wiksell International, Stockholm, Sweden. ISBN 91-22-00783-0.

Patents or patent applications cited: WO 95/07300, US 4,237,224, WO 84/03103.

## Sequence list

10 20 30 40 50 60 70  
5 ACCACCACCACCACCACCACCCCTCTAGTGATGAAGAAAAGAATGATGTGATCAATAATAATCAGTCAATAA  
H H H H H H P S S D E E K N D V I N N N Q S I  
← Pel leader

80 90 100 110 120 130 140  
ACACCGACGATAATAACCAAATAATTAAAAAGAAGAAACGAATAACTACGATGGCATAGAAAAACGCTCAG  
N T D D N N Q I I K K E E T N N Y D G I E K R S

10 150 160 170 180 190 200 210  
AAGATAGAACAGAGTCNACAACAAATGTNGATGAAAACGAAGCAACATTTTACAAAAGACCCCTCAAGATA  
E D R T E X T T N X D E N E A T F L Q K T P Q D

15 220 230 240 250 260 270 280  
ATACTCATCTTACAGAAGAAGAGGTNAAAGAATCCTCATCAGTCGAATCCTCAAATTCATCAATTGATACTG  
N T H L T E E E X K E S S S V E S S N S S I D T

290 300 310 320 330 340 350 360  
CCCAACAACCATCTCACACAACAATAATAGAGAAGAATCTGTTCAAACAAGTGATAATGTAGAAGATTAC  
A Q Q P S H T T I N R E E S V Q T S D N V E D S

20 370 380 390 400 410 420 430  
ACGTATCAGATTTTGCTAACTCTAAAATAAAAGAGAGTAACACTGAATCTGGTAAAGAAGAGAATACTATAG  
H V S D F A N S K I K E S N T E S G K E E N T I

440 450 460 470 480 490 500  
25 AGCAACCTAATAAAGTAAAAGAAGATTCAACAACAAGTCAGCCGTCTGGCTATACAAATATAGATGAAAAA  
E Q P N K V K E D S T T S Q P S G Y T N I D E K

## Sequence list cont.

510 520 530 540 550 560 570  
TTTCAAATCAAGATGAGTTATTAAATTTACCAATAAATGAATATGAAAATAAGGCTAGACCATTATCTACAA  
I S N Q D E L L N L P I N E Y E N K A R P L S T

580 590 600 610 620 630 640  
CATCTGCCCAACCATCGATTAAACGTGTAACCGTAAATCAATTAGCGGCGGAACAAGGTTCTGAATGTTAACC  
T S A Q P S I K R V T V N Q L A A E Q G S N V N

650 660 670 680 690 700 710 720  
ATTTAATTAAAGTTACTGATCAAAGTATTACTGAAGGATATGATGATAGTGAAGGTGTTATTAAAGCACATG  
H L I K V T D Q S I T E G Y D D S E G V I K A H

730 740 750 760 770 780 790  
ATGCTGAAAACCTTAATCTATGATGTAACCTTTGAAGTAGATGATAAGGTGAAATCTGGTGATACGATGACAG  
D A E N L I Y D V T F E V D D K V K S G D T M T

800 810 820 830 840 850 860  
TGGATATAGATAAGAATACAGTTCCATCAGATTTAACCGATAGCTTTACAATACCAAAAATAAAAGATAATT  
V D I D K N T V P S D L T D S F T I P K I K D N

870 880 890 900 910 920 930  
CTGGAGAAATCATCGCTACAGGTACTTATGATAACAAAAATAAACAAATCACCTATACTTTTACAGATTATG  
S G E I I A T G T Y D N K N K Q I T Y T F T D Y

940 950 960 970 980 990 1000  
TAGATAAGTATGAAAATATTAAAGCACACCTTAAATTAACGTCATACATTGATAAATCAAAGGTTCCAAATA  
V D K Y E N I K A H L K L T S Y I D K S K V P N

1010 1020 1030 1040 1050 1060 1070 1080  
ATAATACCAAGTTAGATGTAGAATATAAAACGGCCCTTTCATCAGTAAATAAAACAATTACGGTTGAATATC  
N N T K L D V E Y K T A L S S V N K T I T V E Y

1090 1100 1110 1120 1130 1140 1150  
AAAGACCTAACGAAAATCGGACTGCTAACNTTCAAAGTATGTTTACAAATATAGATACGAAAATCATACAG  
Q R P N E N R T A N X Q S M F T N I D T K N H T

1160 1170 1180 1190 1200 1210 1220  
TTGAGCAAACGATTTATATTAAACNTCTTCGTTATTCAGCCAAGGAACAAATGTAAATATTTTCAGGGAATG  
V E Q T I Y I N X L R Y S A K E T N V N I S G N

## Sequence list cont.

1230 1240 1250 1260 1270 1280 1290  
 GTGATGAAGGTTCAACAATTATAGACGATAGCACAATAATTAAAGTTTATAAGGTTGGAGATAATCAAATT  
 G D E G S T I I D D S T I I K V Y K V G D N Q N

1300 1310 1320 1330 1340 1350 1360  
 TACCAGATAGTAACAGAATTTATGATTACAGTGAATATGAAGATGTCACAAATGATGATTATGCCCAATTAG  
 L P D S N R I Y D Y S E Y E D V T N D D Y A Q L

1370 1380 1390 1400 1410 1420 1430 1440  
 GAAATAATAATGATGTGAATATTAATTTTGGTAATATAGATTCACCATATATTATTAAAGTTATTAGTAAAT  
 G N N N D V N I N F G N I D S P Y I I K V I S K

1450 1460 1470 1480 1490 1500 1510  
 ATGACCNTAATAAGGATGATTACACGACTATACAGCAAACCTGTGACAATGCAGACGACTATAAATGAGTATA  
 Y D X N K D D Y T T I Q Q T V T M Q T T I N E Y

1520 1530 1540 1550 1560 1570 1580  
 CTGGTGAGTTTAGAACAGCATCCTATGATAATAACAATTGCTTTCTCTACAAGTTCAGGTCAAGGACAAGGTG  
 T G E F R T A S Y D N T I A F S T S S G Q G Q G

1590 1600 1610 1620 1630 1640 1650  
 ACTTGCCTCCTGAAAAAATTATAAATCGGAGATTACGTATGGGAAGATGTAGATAAAGATGGTATTCAAA  
 D L P P E K T Y K I G D Y V W E D V D K D G I Q

1660 1670 1680 1690 1700 1710 1720  
 ATACAAATGATAATGAAAAACCGCTTAGTAATGTATTGGTAACTTTGACGTATCCTGATGGAACCTTCAAAT  
 N T N D N E K P L S N V L V T L T Y P D G T S K

1730 1740 1750 1760 1770 1780  
 CAGTCAGAACAGATGAAGATGGGAAATATCAATTTGATGGGGTGCAGGTTCGAC  
 S V R T D E D G K Y Q F D G V Q V D

→  
 Hyc tail

Sequence list. A partial nucleotide sequence of the putative *fig* gene from *S. epidermidis* strain HB and the deduced amino acid sequence. The vector sequences in the junction of the 5'- and 3'-ends are indicated.

### Claims

1. Protein or polypeptide having fibrinogen binding activity, **characterized** in that said protein or polypeptide originates from a coagulase-negative staphylococcal strain.

2. Recombinant DNA molecule containing a nucleotide sequence coding for a protein or polypeptide having fibrinogen binding activity, **characterized** in that said protein or polypeptide originates from a coagulase-negative staphylococcal strain.

3. Plasmid, phage or phagemid containing a nucleotide sequence coding for a protein or polypeptide having fibrinogen binding activity, **characterized** in that said protein or polypeptide originates from a coagulase-negative staphylococcal strain.

4. Micro-organism containing at least one recombinant DNA molecule according to claim

2.

5. Micro-organism containing at least one plasmid, phage or phagemid according to claim

3.

6. Method for producing a fibrinogen binding protein or a polypeptide thereof, **characterized** in that

- at least one recombinant DNA molecule according to claim 2 is introduced in a micro-organism,

- said micro-organism is cultured in a suitable medium,

- the protein thus formed is isolated by chromatographic purification.

7. Method for producing a fibrinogen binding protein or polypeptide thereof, **characterized** in that

- at least one recombinant protein according to claim 2 is expressed on a phage particle,

- said phage particle shows fibrinogen binding activity.



THIS PAGE WAS NOT FURNISHED UPON FILING  
THE INTERNATIONAL APPLICATION

THIS PAGE WAS NOT FURNISHED UPON FILING  
THE INTERNATIONAL APPLICATION

by the DNA sequence according to claim 8.

18. The use of antibodies according to claim 17 for diagnostic purposes.

19. The use of antibodies according to claim 17 for therapeutic and prophylactic purposes.

20. The use of antibodies against the extractable fraction of staphylococci to block the  
5 adherence of staphylococci.

21. The use of antibodies against the native fibrinogen binding protein from staphylococci  
to block the adherence of staphylococci.

22. The use of antibodies against a protein according to claim 1 or parts thereof to block  
the adherence of staphylococci.

23. The use of a fibrinogen binding protein or parts thereof from staphylococci as an  
10 immunogen.

24. The use of a protein according to claim 1 or parts thereof as an immunogen.

25. Vaccine composition including a protein according to claim 1.

26. Vaccine composition including a DNA sequence according to claim 8.

27. Method of active immunisation including the administration of a protein according to  
15 claim 1 to a mammal.

28. Method of active immunisation including the administration of a DNA sequence  
according to claim 8, to a mammal.

29. Method of passive immunisation including the administration of antibodies, raised  
20 against a protein according to claim 1 or against a peptide, encoded by a DNA sequence according  
to claim 8, to a mammal.

Fig. 1

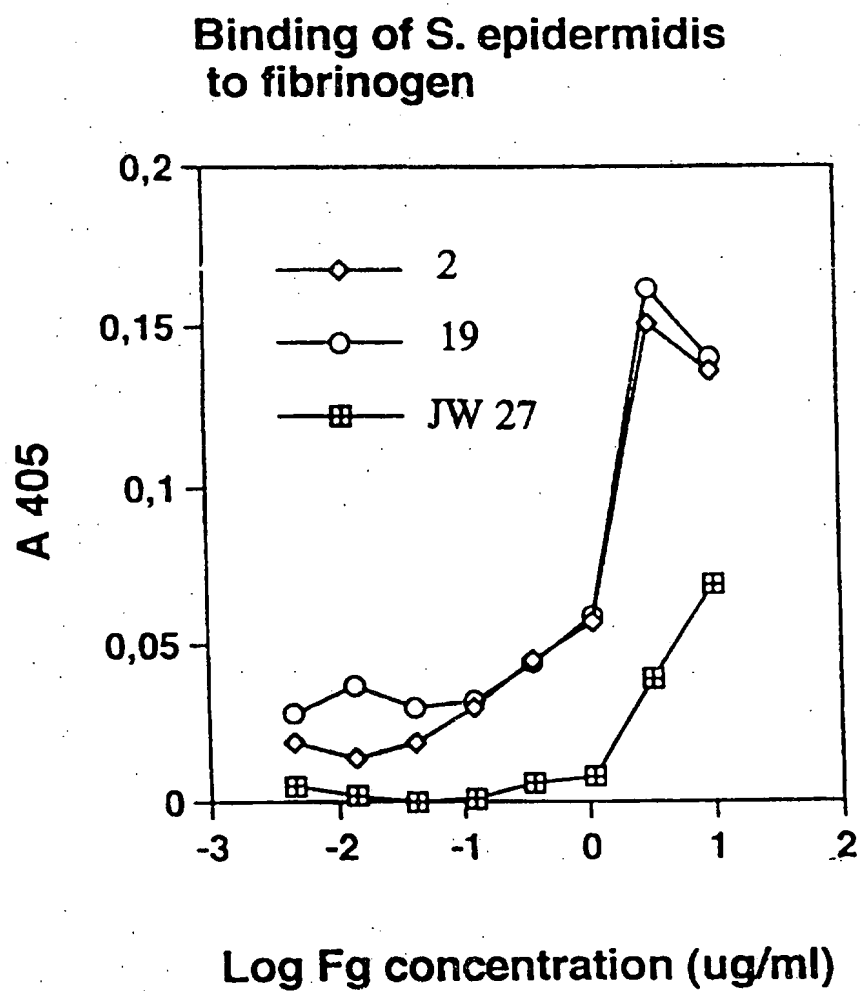
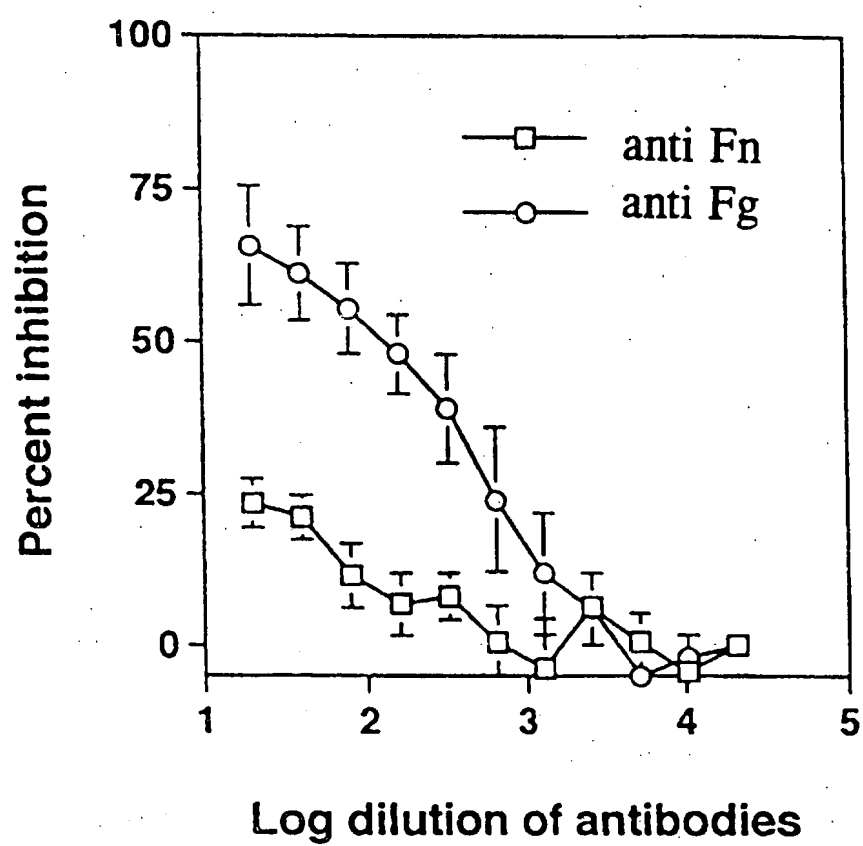


Fig. 2



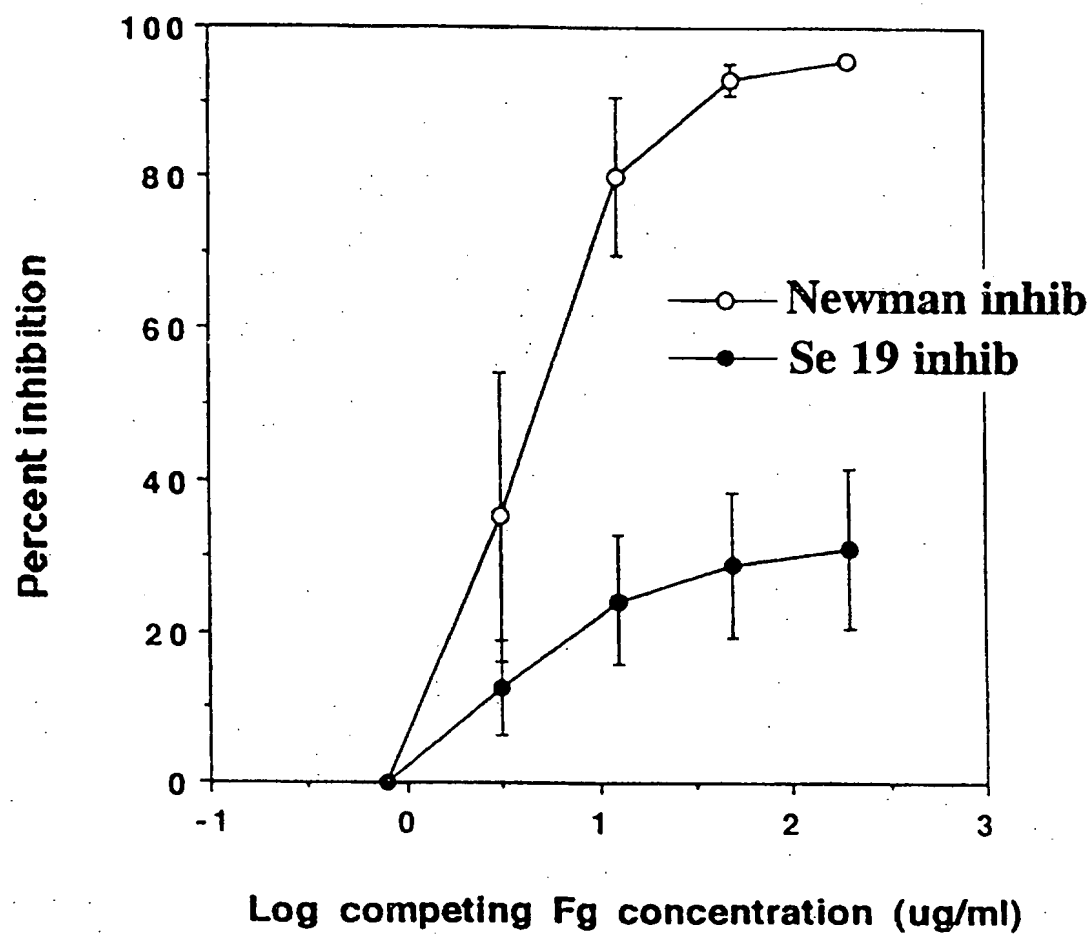
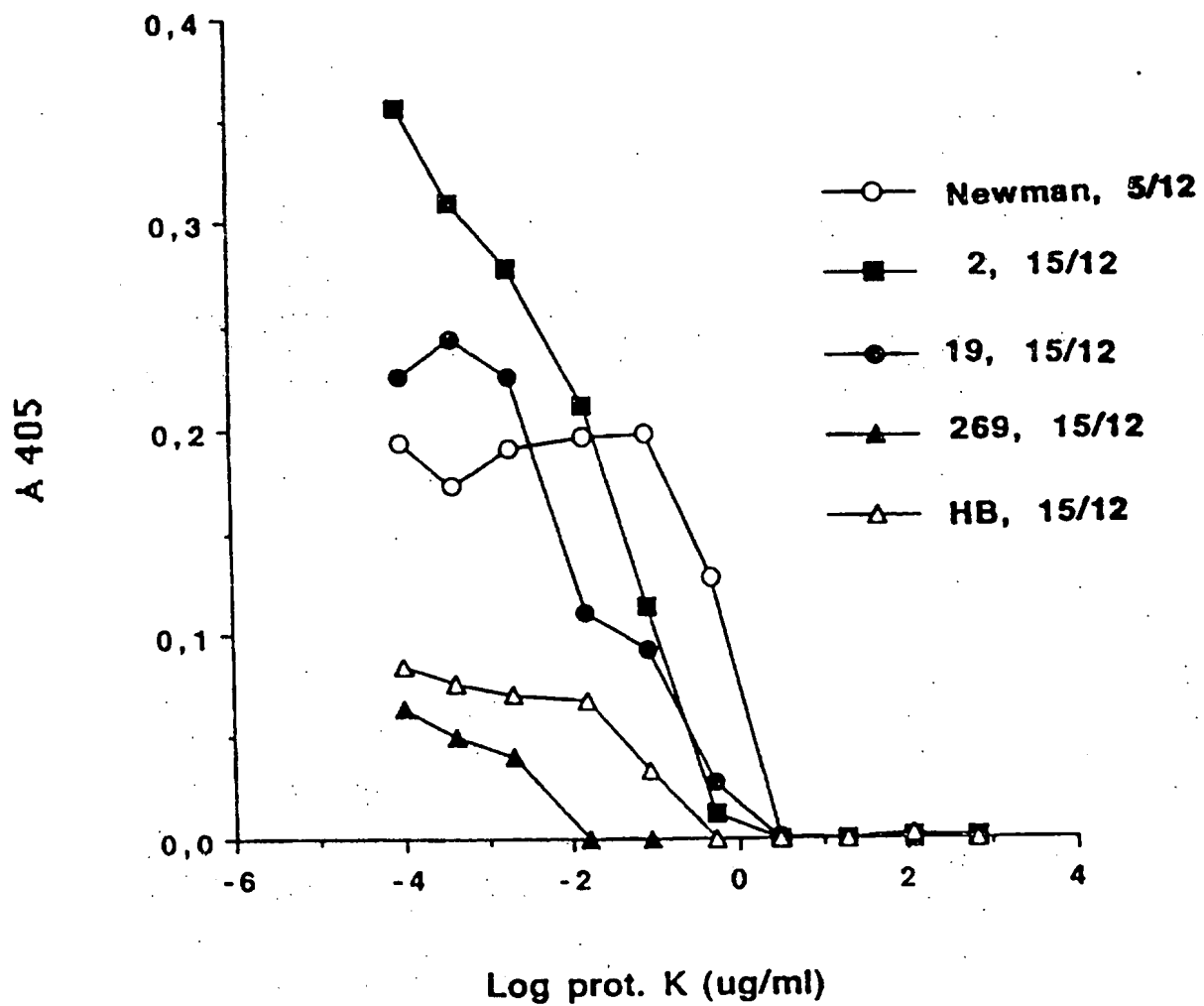
**Fig. 3**

Fig. 4



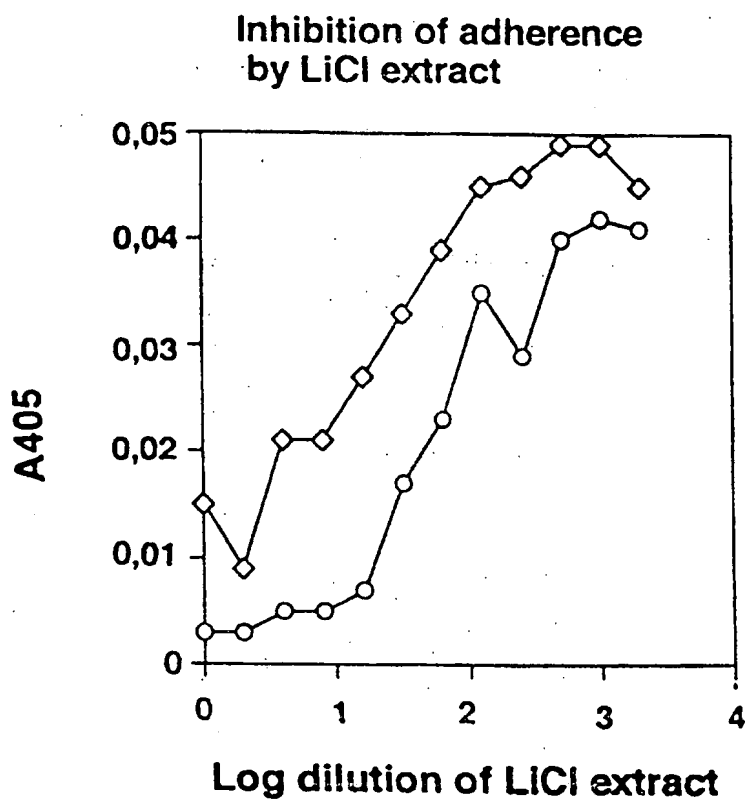
**Fig. 5**



Fig. 6

→S  
TACATTGAAATAGTCAAAGAAAAGGAGTTTTTATGATTAATAAAAAAATAATTTACTAA-60  
M I N K K N N L L -9

CTAAAAAGAAACCTATAGCAAATAAATCCAATAAATATGCAATTAGAAAATTCACAGTAG-120  
T K K K P I A N K S N K Y A I R K F T V -29

GTACAGCGTCTATTGTAATAGGTGCAACATTATTGTTTGGTTTAGGTCATAATGAGGCCA-180  
G T A S I V I G A T L L F G L G H N E A -49

→A  
AAGCCGAGGAGAATTCAGTACAAGACGTTAAAGATTTCGAATACGGATGATGAATTATCAG-240  
K A E E N S V Q D V K D S N T D D E L S -69

ACAGCAATGATCAGTCTAGTGATGAAGAAAAGAATGATGTGATCAATAATAATCAGTCAA-300  
D S N D Q S S D E E K N D V I N N N Q S -89

TAAACACCGACGATAATAACCAAATAATTAAAAAGAAGAAACGAATAACTACGATGGCA-360  
I N T D D N N Q I I K K E E T N N Y D G -109

TAGAAAAACGCTCAGAAGATAGAACAGAGTCAACAACAAATGTAGATGAAAACGAAGCAA-420  
I E K R S E D R T E S T T N V D E N E A -129

CATTTTTACAAAAGACCCCTCAAGATAATACTCATCTTACAGAAGAAGAGGTAAAAGAAT-480  
T F L Q K T P Q D N T H L T E E E V K E -149

CCTCATCAGTCGAATCCTCAAATTCATCAATTGATACTGCCCAACAACCATCTCACACAA-540  
S S S V E S S N S S I D T A Q Q P S H T -169

CAATAAATAGAGAAGAATCTGTTCAAACAAGTGATAATGTAGAAGATTCACACGTATCAG-600  
T I N R E E S V Q T S D N V E D S H V S -189

ATTTTGCTAACTCTAAAATAAAAGAGAGTAACACTGAATCTGGTAAAGAAGAGAATACTA-660  
D F A N S K I K E S N T E S G K E E N T -209

TAGAGCAACCTAATAAAGTAAAAGAAGATTCAACAACAAGTCAGCCGTCTGGCTATACAA-720  
I E Q P N K V K E D S T T S Q P S G Y T -229

ATATAGATGAAAAAATTTCAAATCAAGATGAGTTATTAAATTTACCAATAAATGAATATG-780  
N I D E K I S N Q D E L L N L P I N E Y -249

7/15

## Fig. 6 continued

AAAATAAGGCTAGACCATTATCTACAACATCTGCCCAACCATCGATTAAACGTGTAACCG-840  
E N K A R P L S T T S A Q P S I K R V T -269

TAAATCAATTAGCGGCGGAACAAGGTTTGAATGTTAACCATTTAATTAAAGTTACTGATC-900  
V N Q L A A E Q G S N V N H L I K V T D -289

AAAGTATTACTGAAGGATATGATGATAGTGAAGGTGTTATTAAAGCACATGATGCTGAAA-960  
Q S I T E G Y D D S E G V I K A H D A E -309

ACTTAATCTATGATGTAACCTTTTGAAGTAGATGATAAGGTGAAATCTGGTGATACGATGA-1020  
N L I Y D V T F E V D D K V K S G D T M -329

CAGTGGATATAGATAAGAATACAGTTCCATCAGATTTAACCAGATAGCTTTACAATACCAA-1080  
T V D I D K N T V P S D L T D S F T I P -349

AAATAAAAGATAATTCTGGAGAAATCATCGCTACAGGTACTTATGATAACAAAAATAAAC-1140  
K I K D N S G E I I A T G T Y D N K N K -369

AAATCACCTATACTTTTACAGATTATGTAGATAAGTATGAAAATATTAAAGCACACCTTA-1200  
Q I T Y T F T D Y V D K Y E N I K A H L -389

AATTAACGTCATACATTGATAAATCAAAGGTTCCAAATAATAATACCAAGTTAGATGTAG-1260  
K L T S Y I D K S K V P N N N T K L D V -409

AATATAAAACGGCCCTTTCATCAGTAAATAAAACAATTACGGTTGAATATCAAAGACCTA-1320  
E Y K T A L S S V N K T I T V E Y Q R P -429

ACGAAAATCGGACTGCTAACCTTCAAAGTATGTTTACAAATATAGATACGAAAATCATA-1380  
N E N R T A N L Q S M F T N I D T K N H -449

CAGTTGAGCAAACGATTTATATTAACCCTCTTCGTTATTTCAGCCAAGGAAACAAATGTAA-1440  
T V E Q T I Y I N P L R Y S A K E T N V -469

ATATTTTCAGGGAATGGTGATGAAGGTTCAACAATTATAGACGATAGCACATAATTAAAG-1500  
N I S G N G D E G S T I I D D S T I I K -489

TTTATAAGGTTGGAGATAATCAAATTTACCAGATAGTAACAGAATTTATGATTACAGTG-1560  
V Y K V G D N Q N L P D S N R I Y D Y S -509

AATATGAAGATGTCACAAATGATGATTATGCCCAATTAGGAAATAATAATGATGTGAATA-1620  
E Y E D V T N D D Y A Q L G N N N D V N -529

TTAATTTTGGTAATATAGATTACCATATATTATTAAAGTTATTAGTAAATATGACCCTA-1680  
I N F G N I D S P Y I I K V I S K Y D P -549

8/15

## Fig. 6 continued

ATAAGGATGATTACACGACTATACAGCAAACCTGTGACAATGCAGACGACTATAAATGAGT-1740  
N K D D Y T T I Q Q T V T M Q T T I N E -569

ATACTGGTGAGTTTAGAACAGCATCCTATGATAATACAATTGCTTTCTCTACAAGTTCAG-1800  
Y T G E F R T A S Y D N T I A F S T S S -589

GTCAAGGACAAGGTGACTTGCCTCCTGAAAAAATTTATAAAATCGGAGATTACGTATGGG-1860  
G Q G Q G D L P P E K T Y K I G D Y V W -609

AAGATGTAGATAAAGATGGTATTCAAAATACAAATGATAATGAAAAACCGCTTAGTAATG-1920  
E D V D K D G I Q N T N D N E K P L S N -629

TATTGGTAACTTTGACGTATCCTGATGGAACCTTCAAAATCAGTCAGAACAGATGAAGATG-1980  
V L V T L T Y P D G T S K S V R T D E D -649

GGAAATATCAATTTGATGGATTGAAAAACGGATTGACTTATAAAATTACATTGAAACAC-2040  
G K Y Q F D G L K N G L T Y K I T F E T -669

CTGAAGGATATACGCCGACGCTTAAACATTCAGGAACAAATCCTGCACTAGACTCAGAAG-2100  
P E G Y T P T L K H S G T N P A L D S E -689

GTAATTCTGTATGGGTAAC TATTAATGGACAAGACGATATGACGATTGATAGTGGATTTT-2160  
G N S V W V T I N G Q D D M T I D S G F -709

ATCAAACACCTAAATACAGCTTAGGGAAC TATGTATGGTATGACACTAATAAAGATGGTA-2220  
Y Q T P K Y S L G N Y V W Y D T N K D G -729

TTCAAGGTGATGATGAAAAAGGAATCTCTGGAGTTAAAGTGACGTTAAAAGATGAAAACG-2280  
I Q G D D E K G I S G V K V T L K D E N -749

GAAATATCATTAGTACAAC TACAACCGATGAAAATGGAAAGTATCAATTTGATAATTTAA-2340  
G N I I S T T T T D E N G K Y Q F D N L -769

ATAGTGGTAATTATATTGTTCAATTTTGATAAACCTTCAGGTATGACTCAAACAACAACAG-2400  
N S G N Y I V H F D K P S G M T Q T T T -789

ATTCTGGTGATGATGACGAACAGGATGCTGATGGGGAAGAAGTTCATGTAACAATTACTG-2460  
D S G D D D E Q D A D G E E V H V T I T -809

ATCATGATGACTTTAGTATAGATAACGGATACTATGATGACGAATCGGATTCCGATAGTG-2520  
D H D D F S I D N G Y Y D D E S D S D S -829

ACTCAGACAGCGACTCAGATTCCGATAGTGATTGACTCCGATAGCGACTCCGATTTCAG-2580  
D S D S D S D S D S D S D S D S D S -849

→R

9/15

## Fig. 6 continued

ACAGCGACTCAGATTCAGACAGCGACTCGGATTCTGATAGCGACTCGGATTCAGACAGCG-2640  
 D S D S D S D S D S D S D S D S D S D S -869

ACTCAGACTCAGACAGTGATTCAGATTCAGACAGCGACTCAGATTCCGATAGTGATTCAG-2700  
 D S D S D S D S D S D S D S D S D S D S -889

ACTCAGACAGCGACTCAGATTCTGATAGTGATTCAGACTCAGACAGTGATTCAGATTCAG-2760  
 D S D S D S D S D S D S D S D S D S D S -909

ACAGCGACTCAGATTCCGATAGTGATTCAGACTCAGACAGCGACTCAGATTCCGATAGTG-2820  
 D S D S D S D S D S D S D S D S D S D S -929

ATTCAGACTCAGACAGCGACTCAGATTCTGATAGTGATTCAGACTCAGACAGTGATTCAG-2880  
 D S D S D S D S D S D S D S D S D S D S -949

ACTCAGACAGTGATTCAGATTCCGATAGTGATTCAGACTCCGATAGCGACTCAGACTCGG-2940  
 D S D S D S D S D S D S D S D S D S D S -969

ATAGTGACTCAGATTCTGATAGTGATTCAGACTCCGATAGCGACTCAGACTCGGATAGTG-3000  
 D S D S D S D S D S D S D S D S D S D S -989

ACTCAGATTCTGATAGTGATTCAGACTCAGACAGCGACTCAGATTCTGATAGTGATTCAG-3060  
 D S D S D S D S D S D S D S D S D S D S -1009

ACTCAGTCAGTGATTCAGATTCCGATAGTGATTCAGACTCAGGCAGTGATTCGGATTCCG-3120  
 D S V S D S D S D S D S D S G S D S D S -1029

R←

ATAGTGATTCAGACTCAGACAACGACTCAGATTTAGGCAATAGCTCAGATAAGAGTACAA-3180  
 D S D S D S D N D S D L G N S S D K S T -1049

→M

AAGATAAATTACCTGATACAGGAGCTAATGAAGATTATGGCTCTAAAGGCAGTTACTTG-3240  
 K D K L P D T G A N E D Y G S K G T L L -1069

GAACTCTGTTTGCAGGTTTAGGAGCGTTATTATTAGGGAACGTCGCAAAAATAGAAAAA-3300  
 G T L F A G L G A L L L G K R R K N R K -1089

ATAAAAATTAAATGTTCAAATGAAATTTGTAGAAAGAAGCAGATATGAGATTTGAATAG-3360  
 N K N \* -1092

AAAGTAGATTTAGTCCAACAAATGTAAGATGTTGATTAAACTATAATACTTTCACG-3420

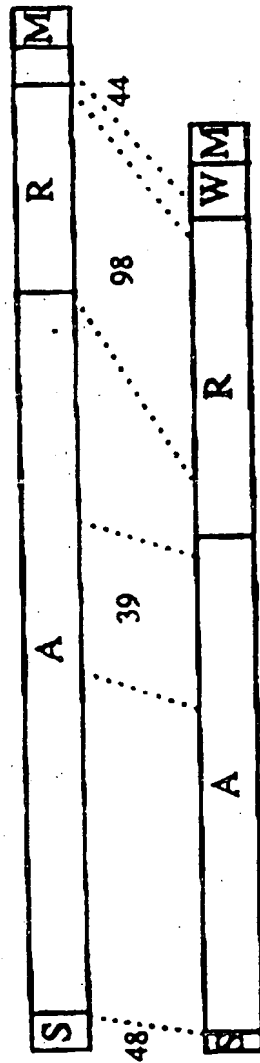
**Fig. 6** continued

TTTATCATATCTTGTGAAAAAGATGATGCAAACAAGGTCATTTCTATTAAAAATGACTTA-3480

AATGTATGATTTTGTAGAGAAACATATACAACCTCACAACTGACAATGATTTAATAGAGGA-3540

ACCGTGAATTTTAAATGAATTCATGGTTCCTTTTATTGAATTAATAAAAAATTCTTTTAT-3600

Fig. 7

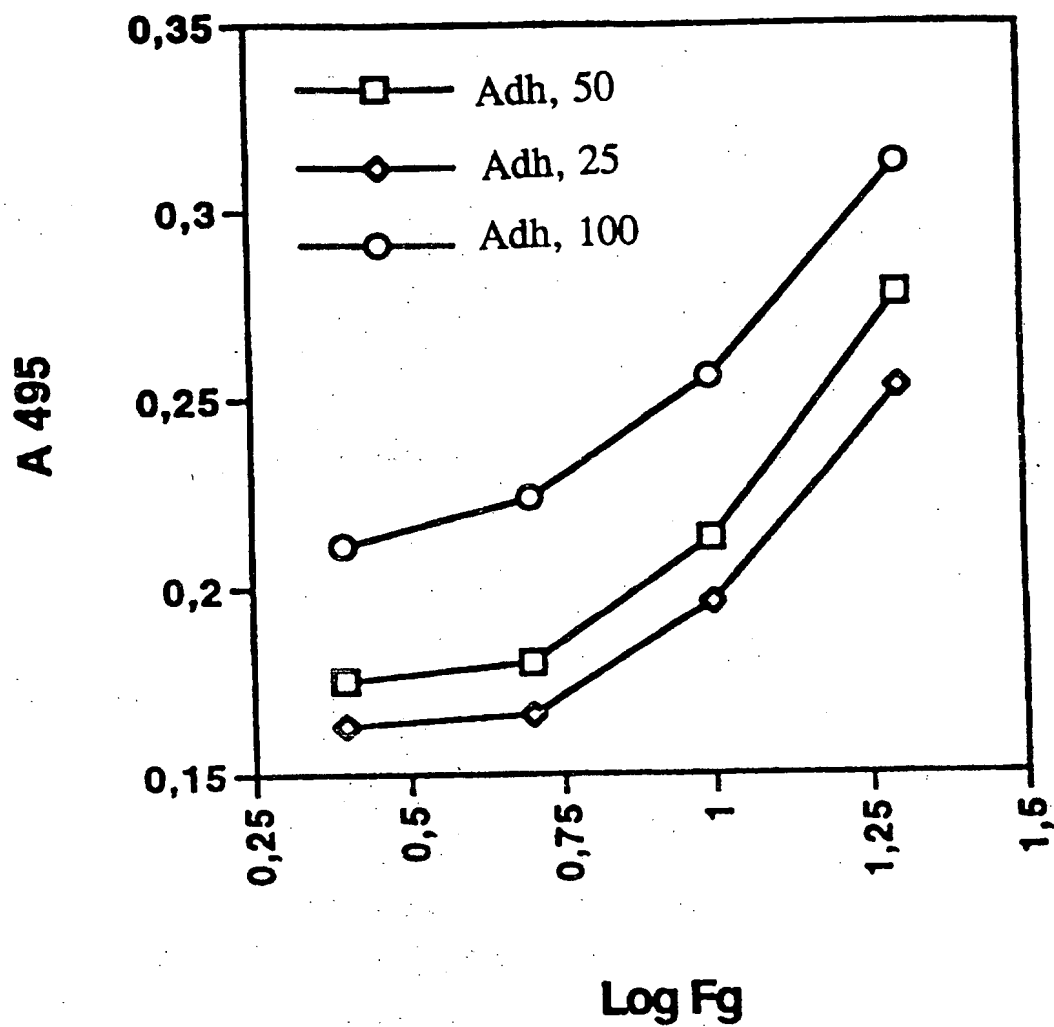


FIG

CIFA

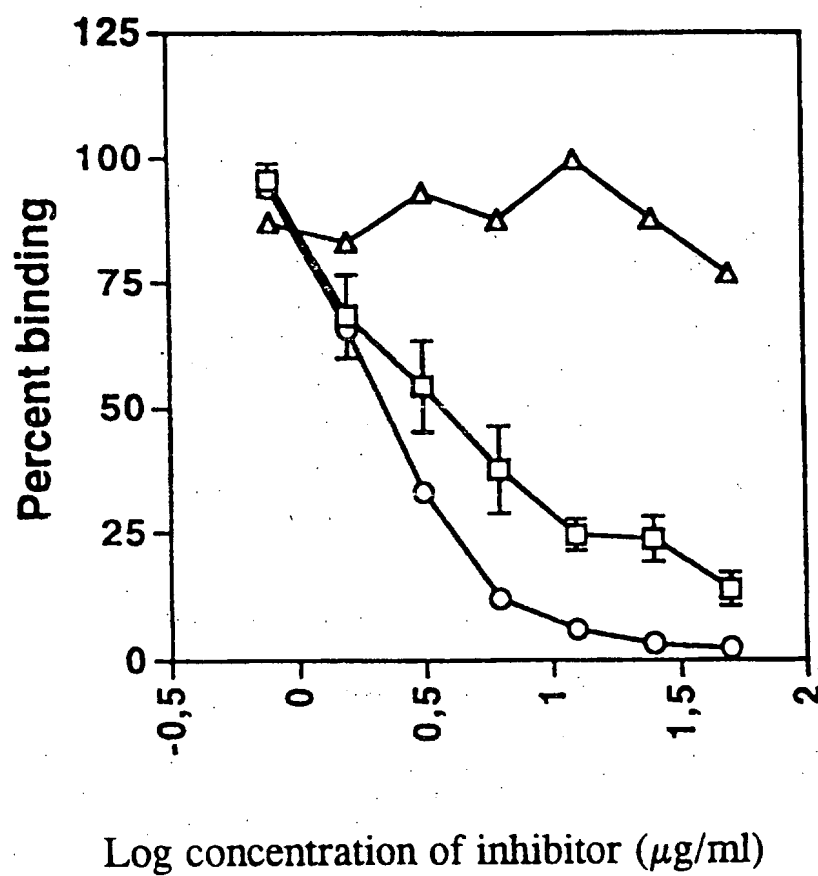
12/15

Fig. 8



13/15

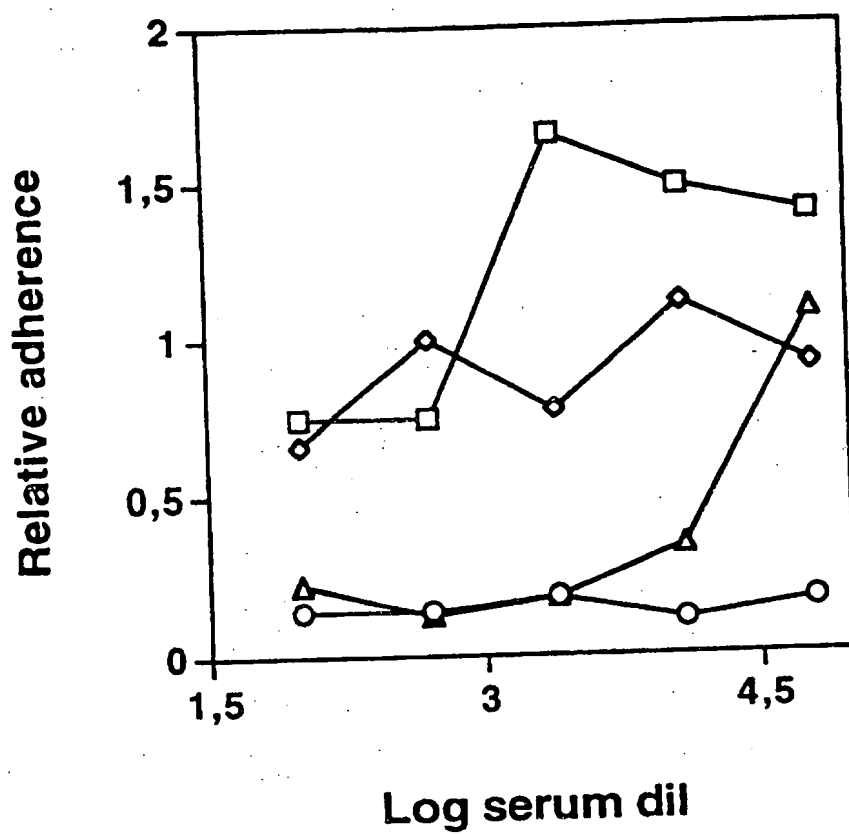
Fig. 9





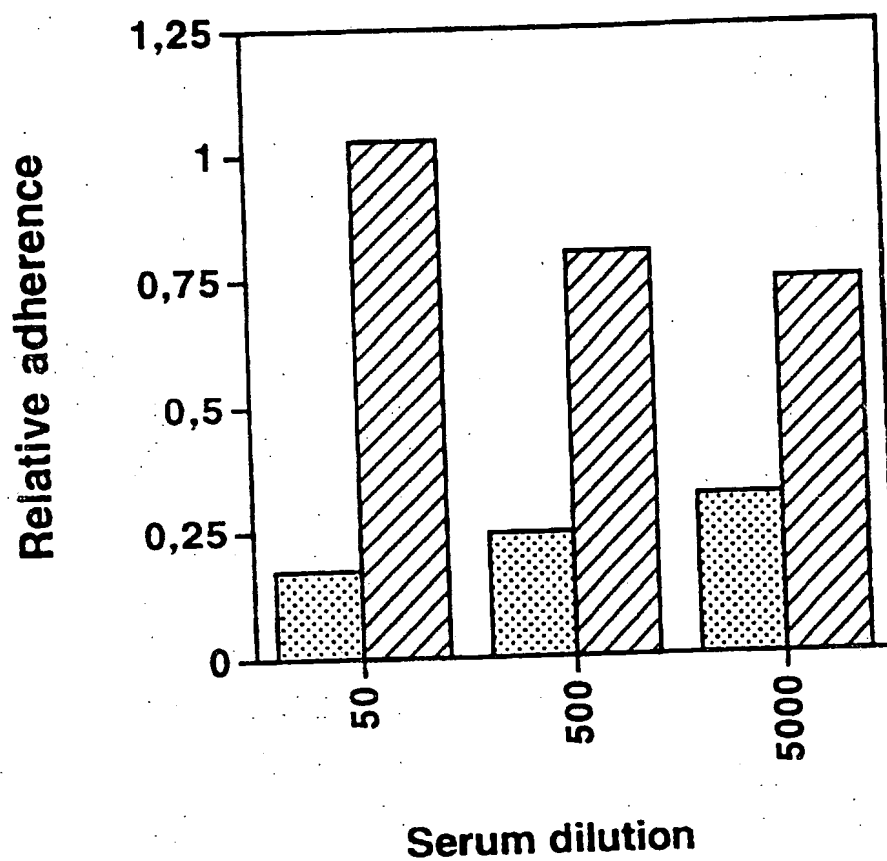
14/15

Fig. 10



- Prelmm No 1
- ◇— Prelmm No 2
- Serum against GST- FIG
- △— Serum against FIG

Fig. 11



▤ Serum against GST-FIG

▨ Pre immune serum

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01091

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/31

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, EMBASE, DIALINDEX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 06062235, Medline accession no. 87072186; Usui Y: "Biochemical properties of fibrinogen binding protein (clumping factor) of the staphylococcal cell surface"; & Zentralbl Bakteriol Mikrobiol Hyg, Sep 1986, 262 (3) p287-97	1
Y		2-7, 20-25, 27, 29

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 Sept 1997

Date of mailing of the international search report

09 -10- 1997

Name and mailing address of the ISA/  
 Swedish Patent Office  
 Box 5055, S-102 42 STOCKHOLM  
 Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz  
 Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01091

C (Continuation): DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 1509169, Biosis accession no. 11033741, Holzhuetter H et al: "Sensitivity and specificity of the clumping factor from a coagulase negative staphylococcus strain against early and late fibrinogen and against fibrin cleavage products in the clumping test"; & VERH DTSCH GES INN MED 79. 1973 (RECD 1974) 1325-1327	1
Y	--	2-7, 20-25, 27, 29
X	WO 9406830 A1 (ALFA-LAVAL AGRI INTERNATIONAL AKTIEBOLAG), 31 March 1994 (31.03.94), page 2, lines 15-27; the claims	20-21, 23
Y	--	2-7, 20-25, 27, 29
A	Molecular Microbiology, Volume 11, No 2, 1994, D. McDevitt et al, "Molecular characterization of the clumping factor (fibrinogen receptor) of Staphylococcus aureus", page 237 - page 248; page 238, left column, lines 43-47	1-7, 20-25, 27, 29
	-----	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01091

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 20-24, 27, 29  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Remark: Although claims 20-24, 27, 29 are directed to methods of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition (c.f. PCT rule 39.1(iv)).
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

01/09/97

International application No.

PCT/SE 97/01091

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9406830 A1	31/03/94	AU 4837193 A	12/04/94
		CA 2124210 A	31/03/94
		EP 0621875 A	02/11/94
		SE 9202720 D	00/00/00
		SE 9302955 D	00/00/00